

OXIDATIVE STRESS BIOMARKERS IN URINE OF PATIENTS WITH HEPATITIS B and C

Abstract

Objectives: The aim of our study is to determine the role of oxidative stress biomarkers on hepatic damage in hepatitis B virus (HBV) and hepatitis C virus (HCV)-infected patients.

Patients and Methods: Forty-eight patients with chronic hepatitis B, 15 patients with chronic hepatitis C and 30 healthy individuals as control group were included in this study. Serum alanine-aminotransferase (ALT) and aspartate aminotransferase (AST) levels, urine oxidative stress biomarkers such as malondialdehyde (MDA) levels, superoxide dismutase (SOD-1) and catalase (CAT) activities were measured.

Results: Urine MDA levels increased in patients of HBV and HCV compared to control group. It was higher in HCV patients than HBV patients ($p < 0.001$). Besides, While CAT and SOD-1 activities were decreased in urine of patients with HCV, they increased in urine of patients with HBV compared to control ($p < 0.05$). Furthermore, urine CAT ve SOD-1 activities in patients of HBV were higher significant statistically than those of the HCV patients ($p < 0.001$).

Conclusion: Increase in urine MDA levels in hepatitis forms may be valuable in monitoring in viral hepatitis cases. Also, we thought that insufficiency of antioxidant barrier in patients with HCV may cause oxidative damage, so antioxidant treatment may useful and should be added to combined therapy for these patients.

Key words: urine, oxidative stress, hepatitis B, hepatitis C, transaminases

HEPATİT B VE C' Lİ HASTALARIN İDRARINDA OKSİDATİF STRES BELİRTEÇLERİ

ÖZET

Amaç: Bu çalışma da, HBV ve HCV enfeksiyonlu hastalarda karaciğer hasarı üzerine oksidatif stres belirteçlerinin rolünün saptanması amaçlandı.

Hastalar ve Yöntemler: Kronik hepatit B'li 48, Kronik hepatit C'li 15 hasta ve 30 sağlıklı birey kontrol grubu olarak çalışmaya alındı. Kontrol ve hasta gruplarının serumlarında alanin-aminotransferaz (ALT) ve aspartat-aminotransferaz (AST) düzeyleri, idrarda katalaz (CAT), süperoksit dismutaz (SOD-1) aktiviteleri ve malondialdehit (MDA) düzeyleri oksidatif stres belirteçleri olarak ölçüldü.

Bulgular: İdrar MDA düzeylerinin HBV ve HCV'li hastalarda kontrol grubuna göre arttığı tespit edildi. HCV'li hastalarda MDA düzeyleri HBV'li hastalara göre daha yüksekti ($p<0.001$). Üstelik, HCV'li hastaların idrarında CAT ve SOD-1 aktiviteleri düşük bulunurken, bu enzimlerin HBV'li hastaların idrarında yüksek olduğu gözlemlendi. Bununla beraber, HBV'li hastalarında idrar CAT ve SOD aktiviteleri HCV'li hastalarından düşük olduğu gözlemlendi.

Tartışma: İdrar MDA düzeylerindeki artış viral hepatit vakalarının takibinde değerli olabilir. HBV'li hastalarda antioksidan enzim aktivitelerindeki artış oksidatif strese karşı hücreyel yanıt olabilir. Bunun yanında, HCV'li hastalarında ki antioksidan bariyer yetersizliği oksidatif hasara neden olabilir, bu yüzden bu hastaların tedavisine antioksidan maddelerin eklenmesinin yararlı olabileceği düşüncesindeyiz.

Anahtar Sözcükler: idrar, oksidatif stres, hepatit B, hepatit C, transaminazlar

48 **Introduction**

49 The free radical, with an unpaired electron, is highly reactive and can damage the cell by
50 peroxidation of phospholipid membranes and oxidation of proteins and DNA, possibly
51 leading to malignant transformation (1). A complex system of neutralizing antioxidants exists
52 in plasma and intra- and extracellular fluids, but an imbalance (oxidative stress) between free
53 radical production and defence can produce cell damage. To prevent destruction caused by
54 oxidative stress, the host uses the antioxidant defence enzymes (2). Antioxidant enzymes such
55 as superoxide dismutase (EC 1.15.1.1, SOD-1) and catalase (EC1.11.1.6, CAT) are closely
56 linked with cellular responses to oxidative stress. SOD-1 (CuZnSOD) is ubiquitously
57 expressed and is a cytosolic scavenger of oxygen free radicals by facilitating the dismutation
58 of oxygen radicals to molecular oxygen and hydrogen peroxide, which in turn is metabolized
59 to harmless water and oxygen by CAT and glutathione peroxidase (GSH-Px). CAT is a heme-
60 containing enzyme, whose subcellular localization is in peroxisomes, and functions in
61 lowering the risk of hydroxyl radical formation from hydrogen peroxide via Fenton reaction
62 (3). Hepatitis C virus (HCV) is the main causative agent of chronic viral hepatitis and
63 hepatitis B virus (HBV), extensively seen throughout the world, can become highly chronic.
64 However, pathogenesis of chronic hepatitis is not known fully yet. It has been suggested that
65 HCV and HBV may cause oxidative stress in infected cell. Lipid peroxidation is one of the
66 reasons for hepatocyte damage and also increases level of malondialdehyde (MDA) which is a
67 biomarker of oxidative stress (4,5). Recently a number of investigators have studied oxidative
68 stress biomarkers levels in serum, plasma and erythrocyte, in patients with hepatitis (6,7). To
69 our of knowledge, none of the previous research investigated oxidative stress biomarkers
70 levels in the urine patients of chronic hepatitis. The present study evaluates, the existence of
71 oxidative stress in urine of patients with HBV and HCV and questions if the disturbances in
72 antioxidant balance are present in these cases. And also it was investigated that if there were

73 differences between hepatitis B and hepatitis C patients of antioxidant enzymes and oxidative
74 stress. Also, we aimed to determine the relationship between serum transaminase levels, and
75 urine oxidative stress biomarkers such as SOD-1, CAT and MDA in patients with HBV and
76 HCV.

77 **Material and Methods**

78 Forty-eight patients with chronic hepatitis B (CHB) (25 males, 23 females; mean age[± SD],
79 36.57 ± 7.20 years), 15 patients with chronic hepatitis C (CHC) (9 males, 6 females; mean
80 age[± SD], 34.46 ± 7.65 years), and 30 healthy individuals as control group (15 males, 15
81 females; mean age[± SD], 35.6 ± 8.0 years; normal medical histories, physical examinations,
82 blood biochemistry, and negative anti-HCV and HBsAg) were included in the present cross-
83 sectional study. Patients were excluded from the study if they had a history of alcohol abuse,
84 other known causes of liver disease (such as metabolic diseases, non-alcoholic steatohepatitis,
85 or any other infectious cause of liver disease), or if the subjects had chronic diseases, such as
86 diabetes mellitus, and cardiac or renal failure. Anti-HCV, HBsAg, anti-HBs, anti-hepatitis B
87 core antigen (HBc; total and anti-HBc immunoglobulin M) were assayed Enhanced
88 Chemiluminescence (VITROS® ECiQ Immunodiagnostic System, Ortho-Clinical
89 Diagnostics, Newjersey, USA). HCV RNA and HBV DNA were investigated using the real-
90 time PCR method (QIAGEN, Rotor-Gene Q, Germantown, USA). Also, alanine
91 aminotransferase (ALT; normal range, 30-65 U/L) and aspartate aminotransferase (AST;
92 normal range, 15-37 U/L) levels were **determined by standard automated techniques** in HBV
93 and HCV-infected patients and controls. The spot urine samples of subjects were collected
94 into 75-mL sterile containers (Kayline Plastics, The barton, South Australia, 5031) which
95 were diluted with 1:50 serum physiologic (0.9% NaCl) for biochemical analysis **and all urine**
96 **samples were stored at -70°C prior to assay**. To control the urine concentration, data were
97 normalized to urine creatinine concentration. Urinary creatinine was measured in spot urine

98 samples by Dade Behring Dimension RXL autoanalyzer (Germany). The study was carried
99 out according to the Guidelines of Local Ethic Committee of The Faculty of Medicine,
100 Kahramanmaras Sutcu Imam University.

101

102 *Biochemical Analysis*

103 SOD-1 activity was measured according to the method described by Fridovich (8).
104 This method employs xanthine and xanthine oxidase to generate superoxide radicals which
105 react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye which was
106 measured at 505 nm. Assay medium consisted of the 0.01 M phosphate buffer, 3-
107 cyclohexilamino-1-propanesulfonicacid (CAPS), buffer solution (50 mM CAPS, 0.94 mM
108 EDTA, saturated NaOH) with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM
109 INT) and 80 U/L xanthine oxidase. SOD-1 activity in urine samples was expressed as U/mg
110 creatinine.

111 CAT activities were determined by measuring the decrease in hydrogen peroxide
112 concentration at 230 nm by the method of Beutler (9). Assay medium consisted of 1 M Tris
113 HCl, 5 mM Na₂EDTA buffer solution (pH 8.0), 1 M phosphate buffer solution (pH 7.0), and
114 10 mM hydrogen peroxide. CAT activity in urine samples was expressed as U/mg creatinine.

115 **Urine MDA levels, as an indicator of lipid peroxidation, in the urine samples were**
116 **determined according to procedure of Ohkawa et al. (10).** The reaction mixture contained 0.1
117 mL sample, 0.2 mL of 8.1 % sodium dodecyl sulphate, 1.5 mL of 20% acetic acid and 1.5 mL
118 of 0.8% aqueous solution of thiobarbituric acid. The mixture pH was adjusted to 3.5 and
119 volume was finally made up to 4.0 mL with distilled water and 5.0 mL of the mixture of n-
120 butanol and pyridine (15:1,v/v) were added. The mixture was shaken vigorously. After

121 centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at
122 532 nm. MDA level was expressed as nmol/mg creatinine.

123 *Statistical analysis:*

124 In statistical evaluation of the data, SPSS 13.0 Windows program was used. Results were
125 considered significant at $p < 0.05$. In comparison of the groups were used One-way ANOVA
126 test. Differences between groups were assessed using the Tukey test.

127

128 **Results**

129 Serum ALT levels in HCV-infected patients were higher than those of the HBV patients and
130 controls. But this change was no significant statistically ($p > 0.05$). However, there was no
131 difference in AST levels between HBV and HCV patients ($p > 0.05$) as shown in Figure 1. In
132 control group, serum AST and ALT levels were found within normal references ranges
133 (Figure 1).

134 While Urine MDA levels increased in patients of HBV and HCV compared to control group.
135 It was two-fold higher in HCV patients than HBV patients ($p < 0.001$) as shown in Figure 2.

136 While CAT and SOD-1 activities were decreased in urine of patients with HCV, they
137 increased in urine of patients with HBV compared to control ($p < 0.05$). Furthermore, urine
138 CAT ve SOD activities in patients of HBV were higher significant statistically than those of
139 the HCV patients ($p < 0.001$) as shown in Figures 3 and 4.

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144 **Discussion**

145 The protective antioxidant mechanisms are complex and multifactorial. The susceptibility of
146 cells to oxidative stress is a function of the overall balance between the degree of oxidative
147 stress and the antioxidant defense capability. Oxidative stress is involved in a number of
148 pathological conditions such as inflammation and cancer which has been detected in almost
149 all clinical and experimental conditions of the chronic liver diseases (4,11). Although the
150 main role of immunological mechanisms in pathogenesis of chronic viral hepatitis B and C
151 were demonstrated, researchers also concentrate on the problem of oxidative stress in
152 pathology of the diseases. Ideally, markers of lipid peroxidation and antioxidants should be
153 measured in hepatic tissue (rather than in blood and urine) to reflect the true state of oxidative
154 stress in the liver but ethical and practical considerations make this very difficult for research
155 purposes. Needle liver biopsy carries a significant risk of morbidity and even mortality and
156 provides very limited amounts of material (<5 mg generally). It is impossible, with current
157 techniques, to perform on liver biopsy material the multiple biochemical estimations which
158 we have carried out in urine; as this study was designed to establish a baseline for long term
159 monitoring of oxidative stress in this condition, and since repeated biopsy at short intervals to
160 follow the course of progression or treatment can not be justified, measurement of **urine**
161 **biomarkers** of oxidative stress, interpreted with caution, offers the only practical option at
162 present. There are some studies in the literature that investigated antioxidants and oxidative
163 stress in plasma, serum and erythrocyte, in patients with hepatitis. De Maria et al. showed that
164 MDA, a product of polyunsaturated fatty acid peroxidation, was elevated in the liver and the
165 blood (12). Paradis et al. also demonstrated MDA-protein adducts immunohistochemically in
166 infected liver tissue (13). Boya et al. showed that the peripheral blood mononuclear cells from
167 patient of CHC had increased MDA concentration (14). Romero et al. showed higher serum
168 MDA values in chronic hepatitis C patients than healthy subjects before the interferon

169 treatment (15). In the present study, we demonstrated that MDA, one of the most reliable
170 markers for lipid peroxidation, was increased in urine of patients with chronic viral hepatitis
171 in association with serum ALT levels. Increase in urine MDA levels in hepatitis forms may be
172 valuable in monitoring in viral hepatitis cases.

173 Levels of antioxidant enzymes, such as SOD-1 and CAT, are closely linked with cellular
174 responses to various oxidative stress. Chrobot et al. demonstrated that SOD-1 and CAT
175 activities decreased both in group of children with CHC, and CHB (16). Loginov et al. studied
176 antioxidant system in adults with chronic active hepatitis, and demonstrated SOD-1 decrease
177 correlating with severity of inflammatory process (17). **Ciragil et al. showed that antioxidant**
178 **enzyme activities (SOD-1, CAT) decreased in serum of patients with HCV compare to control**
179 **group (18).** Study by Yasuyama et al. showed decrease of SOD-1 levels in liver tissue of
180 patients with acute, and chronic hepatitis accompanied by fatty degeneration while comparing
181 with patients with the liver inflammatory diseases of different etiology (19). In the current
182 study we demonstrated that CAT and SOD-1 activities are decreased in urine of patients with
183 chronic hepatitis C. The capacities of antioxidant enzymes were decreased in patients with
184 CHC; thereby we thought that there may be an oxidative damage in these patients. When the
185 activities of these antioxidant enzymes were insufficient, the organism is not capable to
186 neutralize excessive ROS, and hepatocyte lesion occurs as a consequence. It probably
187 decreased the antioxidant barrier efficiency in studied CHC patients. The reduction in the
188 amount of SOD-1, and CAT reflects both a decrease in the synthesis capacity of liver, and
189 the antioxidant defense power of the patients with CHC. It can be argued that increased lipid
190 peroxidation is caused by the inflammation related to viral infection and decreased the
191 antioxidant levels may be an early marker of the oxidative stress. Lipid peroxides formed can
192 be chemotactic for the neutrophils causing increased inflammation, which further drives
193 oxidant-mediated injury in the liver (20). The results presented confirm the involvement of

194 the oxidative stress as a part of pathophysiology of CHC. Thus, our findings support the
195 existence of the oxidative stress in patients with chronic HCV infection and are in agreement
196 with the studies mentioned above.

197 In several studies (16, 20-22), increase in oxidative components or decrease in antioxidants or
198 both have been reported in subjects with either acute or chronic HBV or HCV infection.
199 **Osman et al. (21) reported that an increase in oxidative stress markers (MDA, nitric oxide)**
200 **and a decrease in antioxidant enzyme activities (SOD-1, GSH and GSH-Px were observed in**
201 **serum of patients with viral hepatitis.** Total antioxidant capacity in either acute or chronic
202 HBV infection was measured in only in study of **Irshad et al (11)**. The remaining was used
203 individual antioxidants measurement to assess antioxidant response of the organism. At the
204 same way, simultaneously measurement of the oxidants and antioxidant components of the
205 plasma in CHB infection was performed in only at study of Demirdag et al (22). The
206 information in the literature about the antioxidant components in subjects with cirrhosis due to
207 HBV infection is limited. Irshad et al (11) found that total antioxidant capacity of subjects
208 either with cirrhosis due to HBV infection or other liver disease due to viral etiology is either
209 comparable to or higher than control. Our results showed that urine CAT and SOD-1 were
210 significantly increased in CHB patients, so there may be a response to oxidative stress in
211 patients with CHB.

212 In a conclusion, according to the results of this study, it might be thought that urine
213 MDA level might be a marker of hepatocellular damage in CHB and CHC patients. And also
214 ROS take part in pathogenesis of CHC as they decrease antioxidant enzymes activities.
215 Therefore we thought that, in the treatment of chronic hepatitis B and C, antioxidant treatment
216 may useful and should be added to combined therapy for these patients. Also, further in-vitro
217 and in-vivo studies are required to enlighten the role of antioxidants on HBV disease
218 progression and treatment.

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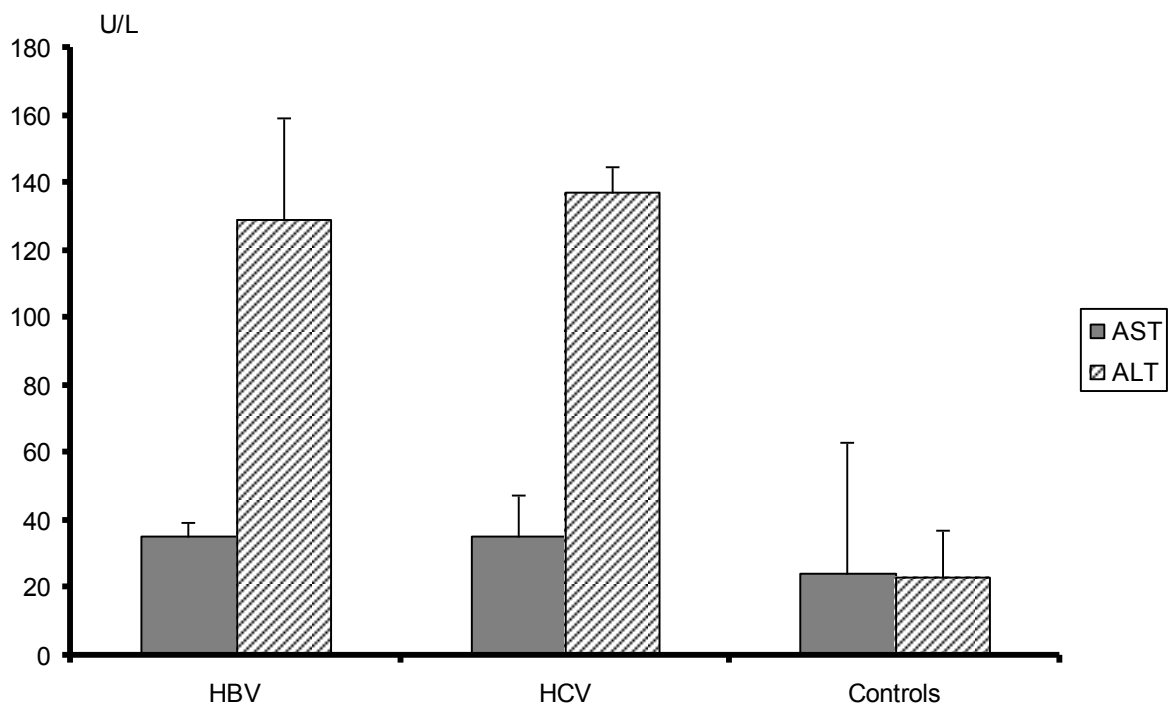
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277 Figure 1. Serum ALT and AST levels in HBV, HCV patients and controls.

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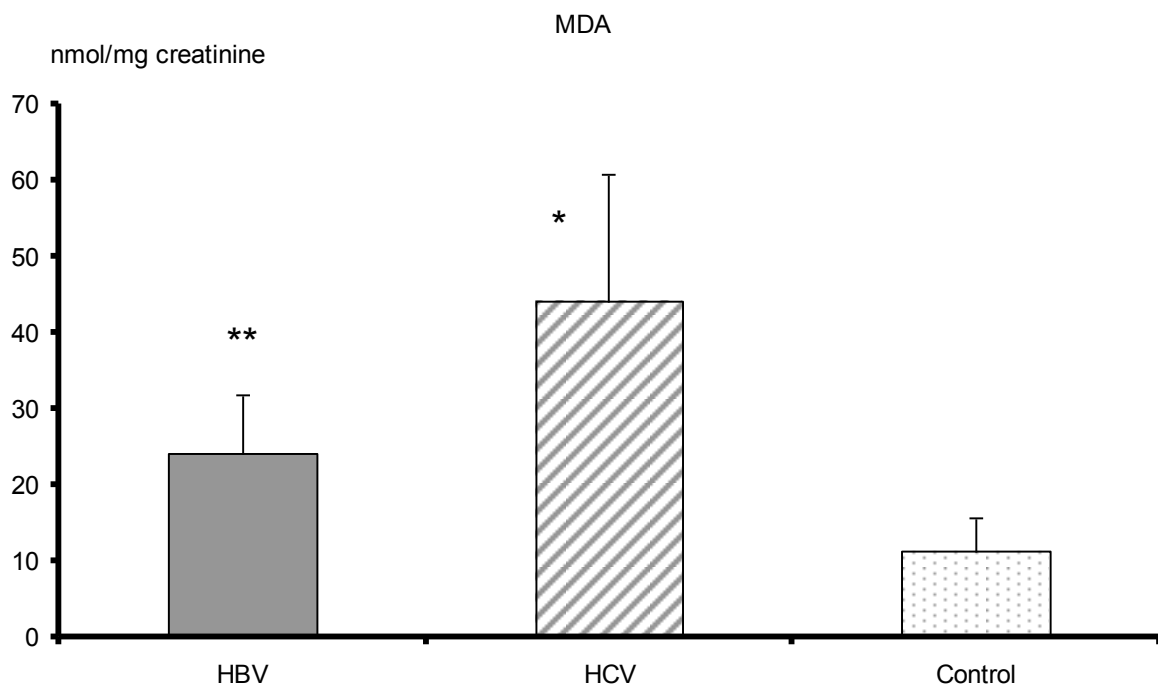
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286 Figure 2. Urine MDA levels in HBV, HCV and control groups

287 * $p < 0.05$, found statistically significant than control and HBV groups

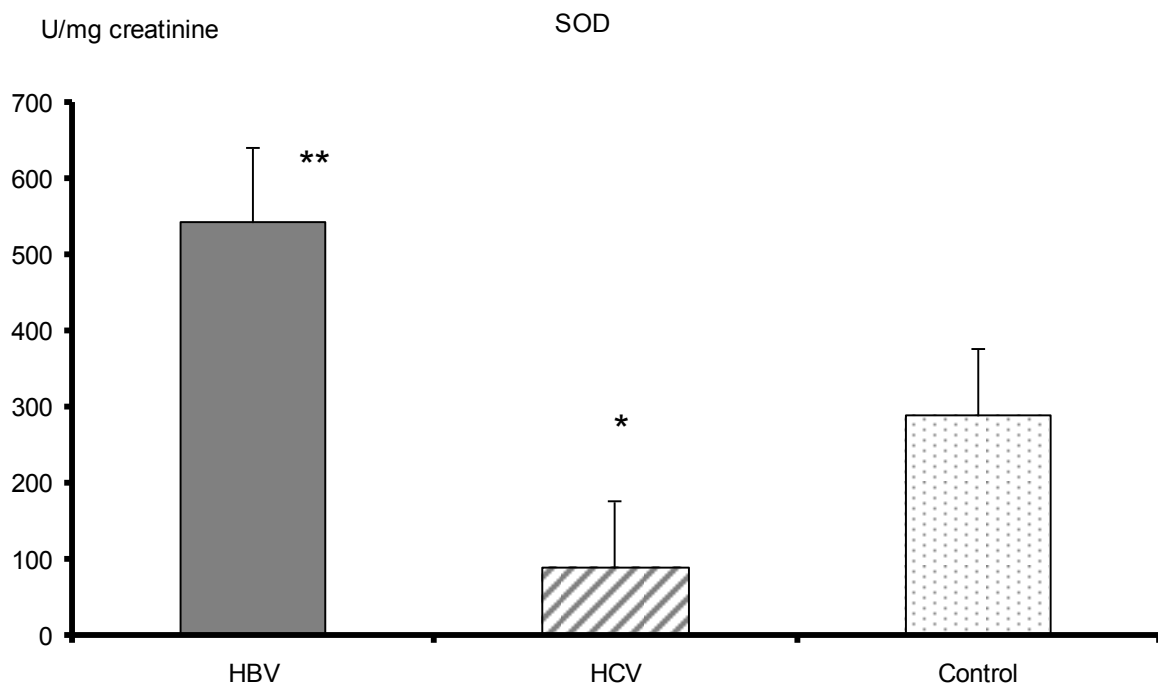
288 ** $p < 0.05$, found statistically significant than control

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294 Figure 3. Urine SOD-1 activities in HBV, HCV and control groups

295 * $p < 0.05$, found statistically significant than control and HBV groups

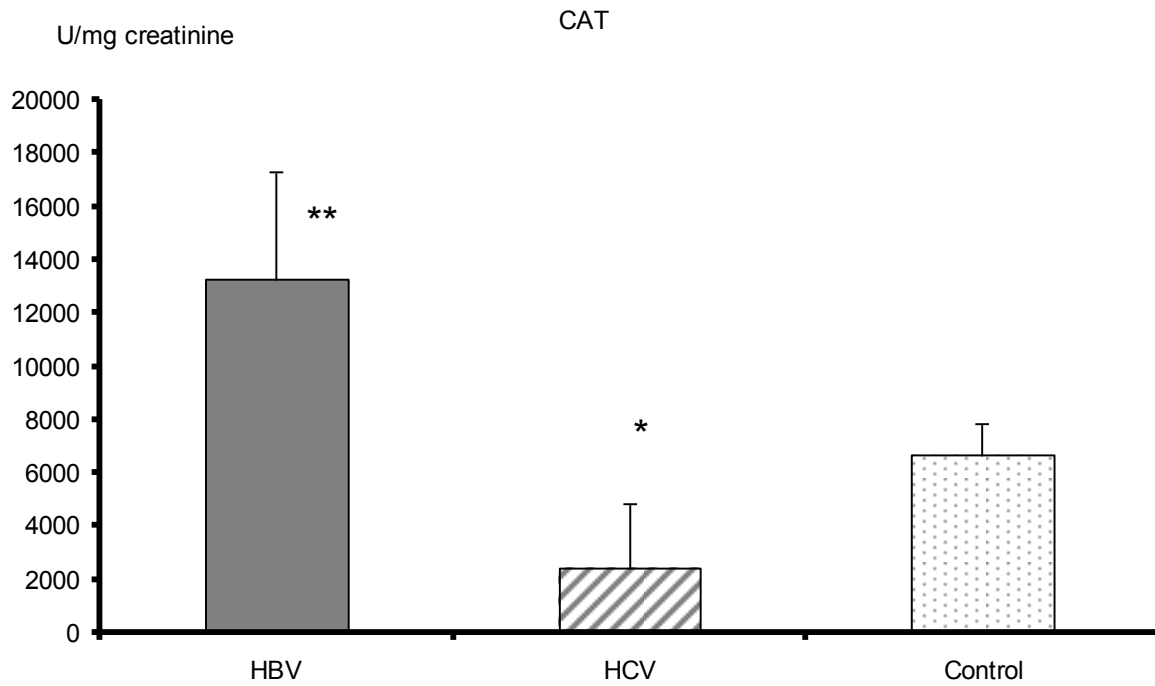
296 ** $p < 0.05$, found statistically significant than control

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302 Figure 4. Urine CAT activities in HBV , HCV and control groups

303 *p<0.05, found statistically significant than control and HBV groups

304 **p<0.05, found statistically significant than control

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